Noel Wat
Deans Scholars Honor Thesis
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I. Effects of eATP on *Arabidopsis* Root Hair Growth

Abstract

Recent research has suggested that ATP may not only serve as an energy storage molecule, but when present outside of the cell, can also serve as a growth regulator in plants. This project describes a study on the signal transduction pathway by which extracellular ATP (eATP) influences root hair growth in *Arabidopsis thaliana.* High concentrations of ATPγS or ADPβS (≥ 150 μM) can inhibit root hair growth, while low concentrations of ATPγS or ADPβS (15-30 μM) will promote growth when the root hairs are pre-washed. The hairs are washed to remove endogenous levels of eATP. This may hint that there is an optimal concentration of eATP needed for root hair growth. Additionally, ectoapyrases, which regulate the concentration of extracellular nucleotides, can be chemically inhibited to reduce root hair growth.

Introduction

Adenosine triphosphate (ATP) is commonly known as the main energy source for cells. However, in animal cells it is well established that ATP located outside of the cell can be used as a signaling agent. More recently, research performed on *Arabidopsis* has provided evidence that extracellular ATP (eATP) has the same function in plants (Demidchik et al., 2003; Jeter et al., 2004). Experiments performed by Kim et al. (2006) using a hybrid luciferase, cellulose binding domain protein which binds to cellulose in the extracellular matrix (ECM), were used to report where eATP was present in plants. The amount of light given off was proportional to the amount of ATP present. Kim et al. (2006) found that eATP is present at the highest concentrations outside cells and tissues that are actively growing, especially around the tip of the root hair. This result suggests that as a plant cell grows, secretory vesicles that deliver wall materials needed for growth also release ATP to the extracellular matrix, thereby causing a buildup of ATP. Relevant, to the described experiment, Kim et al. (2006) found that elongating root hairs release ATP at their tips.

Enzymes called ectoapyrases are used to regulate the level of extracellular nucleotides by hydrolyzing eATP and eADP. Ectoapyrase inhibitors such as apyrase inhibitor #13 and NGXT 191 will retard the rate of eATP destruction, thereby increasing the concentration of eATP around the root hair cells. Additionally, Wu et al. (2007) showed that the two main ectoapyrases in *Arabidopsis*, AtAPY1 and AtAPY2, are required for normal growth of roots, shoots, and pollen tubes. Additionally, treatment of roots with apyrase inhibitors caused inhibition of root hair growth (Clark et al., In review). These results illustrate the importance of ectoapyrases in regulating eATP for growth in single-cell polar cells.

Arabidopsis root hairs were chosen as the model system because *Arabidopsis* plants are small and grow quickly, making it suitable for study in the lab environment. Moreover, the *Arabidopsis* genome has been completely sequenced. More importantly root hairs serve as a simple single-cell model that grows in a polar fashion. Thus, the effects of eATP and various other molecules can be easily reflected in the growth rates of the root hairs. Additionally, non-hydrolyzable forms of ATP and ADP, ATPγS and ADPβS were used to ensure that any observed changes in growth are due to ATP acting as a signaling molecule and not as an energy source.

Reichler et al. (2009) found that pollen tube elongation can be inhibited with concentrations of 150 μM ATPγS. Additionally, 200 μM AMPS did not inhibit growth. They hypothesized that AMPS is structurally different enough that it does not act as a signaling molecule like ATP. Since pollen tubes are also single-cell models of growth, similar results might be found in an analysis of root hair growth. To test this possibility, a dose response curve for high concentrations of ADPβS was performed. In order to test the effect of low concentrations of ADPβS without the presence of endogenous eATP, root hairs were washed with MS buffer solution to remove pre-existing ATP. Such a wash, if done carefully and gently should not adversely affect the environment of the root hairs. Moreover, ATP is soluble and will easily dissolve in the MS buffer solution.

Nitric oxide (NO) may also play a role downstream of eATP in root hair growth. This is evidenced in that extracellular nucleotides can induce NO production in tomato cell culture (Foresi et al., 2007) and *Salvia* hairy root cultures (Wu and Wu, 2008). Moreover, Reichler et al. (2009) found that NO agonists alter the effects of ATPγS on pollen germination by reducing the concentration of ATPγS needed to inhibit pollen germination. On the other hand, NO antagonists will block the effects of ATPγS on pollen germination and elongation, thereby

making ATPγS less potent. More specifically, addition of 100 μM ODQ, a guanylate cyclase inhibitor, was able to block the inhibition of pollen germination by 250 μM ATPγS. However, treatment with this concentration of ODQ by itself did not have an effect on pollen germination.

Materials and Methods

All *Arabidopsis thaliana* seeds used in this study were of Columbia (Col-0) ectotype. Seeds were sterilized as described by Tang et al. (2003). *Arabidopsis* seeds were surface sterilized in 20% commercial bleach for 10 min and subsequently rinsed with autoclaved distilled water six times. The seeds were then vernalized in the dark at 4°C in distilled water for approximately 3 days. Next, the seeds were planted on cellophane membrane on solidified Murashige and Skoog (MS) with 1% agar. The MS media was raised to pH 5.7 with KOH and autoclaved for sterility. See appendix for the composition of the MS media. Planted seeds were placed upright in a culture chamber and grown at 23° C under 24-h fluorescent light for approximately 3.5 days. After 3.5 days the seedlings were transferred to a new treatment plate containing the chemical to be tested.

Treatment plates were made using the same agar composition as those used for planting. These plates were usually made either the day before or on the same day of the experiment. The desired chemical was added to the agar shortly before solidification when the agar temperature was near 40°C. For experiments using ADPβS and ATPγS, 20mM stocks were always used. This stock concentration helped ensure that not too much or too little volume of stock solution would be added, which aided pipetting accuracy without greatly changing the composition ratios of the agar. In experiments that tested the role of NO in eATP signaling, 1H-[1,2,4]Oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was dissolved in DMSO with a final

concentration of 0.1% DMSO in the MS agar plate. Therefore, 0.1% DMSO was also added to other treatment plates used in the same experiment.

Before transferring the seedlings to the treatment plate, some seedlings were washed to remove the natural levels of eATP in the extracellular matrix. Roots were gently submerged in a liquid MS buffer solution and the solution was wicked off from the hypocotyls each time using Kimwipes. The MS buffer solution was prepared the same as the agar for planting sans agar. Plates were tilted vertically with the primary root pointing up so that the liquid would aggregate near the hypocotyls, where it would be removed. Care was taken to not disturb the primary root or root hairs to prevent any type of touch stimulation. This wash procedure was performed three times before transferring the seedlings to the treatment plate.

Seedlings were transferred to the experimental plate by gently lifting the cellophane with tweezers and placing it atop the experimental plate. Tweezers were also used to remove any air bubbles that may prevent contact between the cellophane and agar by guiding air bubbles to the edge of the cellophane.

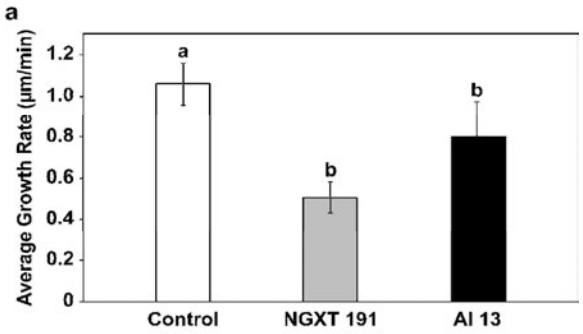
Pictures of the root tip and root hairs were taken using Motic Images Plus 2.0 under 40X magnification at time zero and at 60 minutes afterwards. Root hair lengths were then measured using ImageJ. Only root hairs near the root tip that were ≤ 150 μm in length at the time zero were measured. Additionally, any root hair growth rates less than 0.02 μm/min were also omitted to account for re-measuring errors.

Results

Apyrase inhibitors inhibit root hair growth

Application of 2.5 μg/ml of either apyrase inhibitors, NGXT191 and AI 13, significantly inhibited root hair growth.

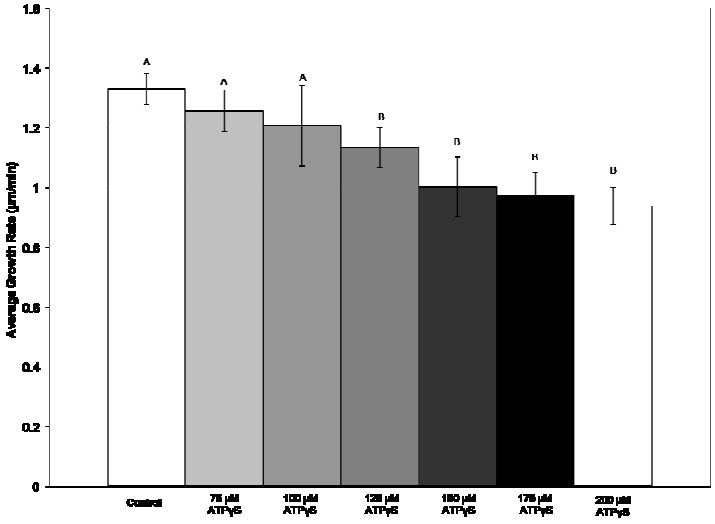
Figure 1. Inhibition of root hair growth by apyrase inhibitors



High concentrations of ATPγS inhibit root hair growth

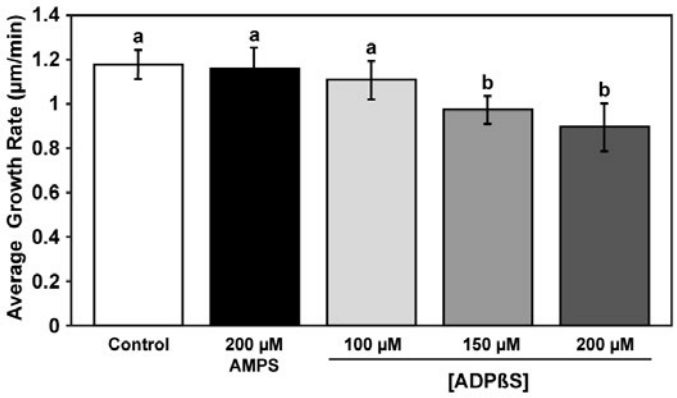
Figure 2 gives the dose response curve of 75 μM to 200 μM ATPγS. As seen, concentrations of ATPγS greater than 150 μM inhibits growth of *Arabidopsis* root hairs.

Figure 2. ATPγS dose response curve



Similar to that found in pollen tubes, concentrations of ADPβS greater than 150 μM were found to inhibit root hair growth. Additionally, 200 μM AMPS did not inhibit root hair growth; this indicates that AMPS may not act like eATP as a signaling agent.

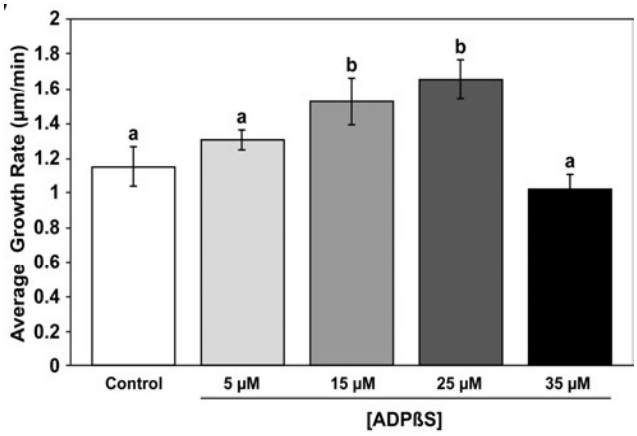
Figure 3. High concentrations of ADPβS inhibit growth



Low concentrations ADPβS promote growth in pre-washed root hairs

Treatment of root hairs with 10 to 30 μM ADPβS had no effect on their average growth rates. However, when *Arabidopsis* seedlings were pre-washed with MS buffer solution to remove the natural endogenous eATP, low concentrations of 15 μM and 20 μM ADPβS were shown to promote root hair growth.

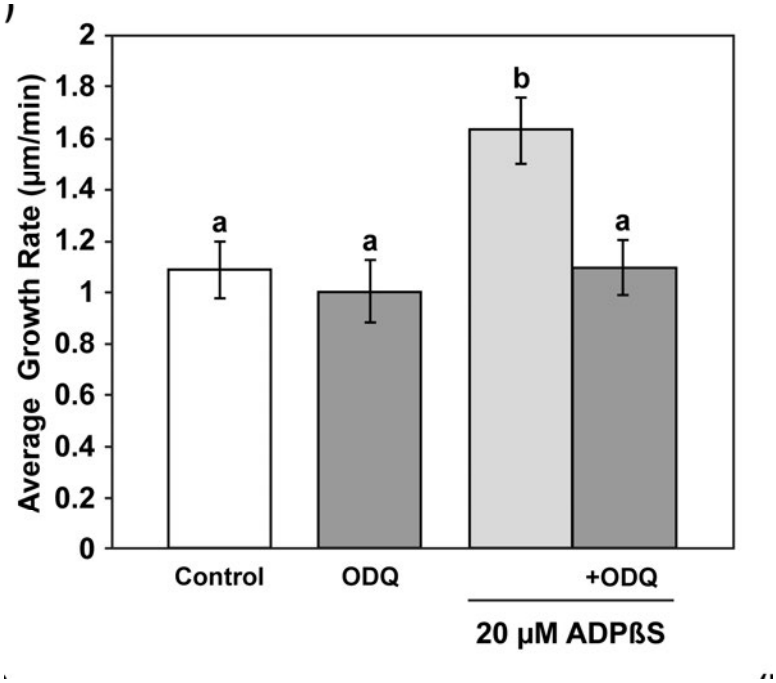
Figure 4. Dose response curve of ADPβS on pre-washed root hairs



Reversal of root hair growth promotion with ODQ

On prewashed root hairs, 20 µM ADPβS effectively promoted the root hair growth rate, while 25 µM ODQ did not have an effect on root hair growth alone. Co-incubation of 20 µM ADPβS and 25 µM ODQ did not show promotion of growth. This indicates that ODQ blocked the promotion of growth by ADPβS.

Figure 5. ODQ can reverse promotion of growth.



Discussion

The methods used to study the effects of *Arabidopsis* root hair growth as outlined are unique as they report changes in growth rate over one hour time periods. Using cellophane to transfer the seedlings onto the treatment plate provided a quick and simple way to allow the chemical of interest to come into contact with the seedling without major disturbance of the root tissue. Cellophane is also useful as it has relatively large pores which facilitate the rapid diffusion of the chemical to the root hairs to influence growth.

Wu et al. (2007) found that the inhibition of ectoapyrases in *Arabidopsis* pollen tubes resulted in an inhibition of elongation accompanied by an increase in eATP. Similarly, application of 2.5 μg/mL of either apyrase inhibitors NGXT191 or AI 13 significantly inhibited root hair growth. This illustrates the importance of ectoapyrase in regulating the concentration of eATP. Thus, inhibition of ectoapyrase causes an increase in eATP which in turn will inhibit growth. This view is supported by the results that high concentrations of ADPβS or ATPγS at or above 150 μM significantly inhibited root hair growth.

Additionally, the wash method used to remove endogenous eATP promoted root hair growth in conjunction with application of 15-25 μM ADPβS. This suggests that there may be an optimal range of extracellular nucleotides to induce root hair growth. Perhaps the concentration of extracellular nucleotides acts as a feedback regulator for root hair growth. When concentrations of eATP are too low, the root hair will not grow rapidly there is no signal for growth. However, concentrations of 15-25 μM ADPβS may jump start growth by signaling that enough cell material is present for growth. On the other hand, when concentrations of extracellular nucleotides are at or above 150 μM ADPβS, the root hair will stop growing as the cell detects sufficient growth.

Ectoapyrase inhibitors inhibited root hair growth by up to 60%, meanwhile high concentrations of ADPβS or ATPγS inhibited root hair growth by only 20-40%. This may indicate that ectoapyrases may have additional effects on root hair growth other than regulation of eATP levels. Application of ADPβS or ATPγS may only play a role in receptor-mediated signaling in *Arabidopsis* root hairs. On the other hand, application of low concentrations of ADPβS or ATPγS on pre-washed root hairs resulted only in 20-30% promotion of growth. This may be because root hairs are already growing close to optimal growth rates. Additionally, the

touch stimulation from washing the root hairs may have slightly decreased growth rates, as shown by the lower growth rates of pre-washed control plates.

Jeter et al. (2004) and Weerasinghe et al. (2009) have demonstrated that mechanical or touch stimulation may cause ATP to be released into the extracellular matrix. This release of ATP may inhibit growth. Transfer of the seedlings via cellophane and washing the root hairs may have induced a touch response in the *Arabidopsis* seedlings. However, because all the plates were transferred and appropriate controls were used when washing roots, the effects of touch stimulus may be negated.

The experiments performed used either ADPβS or ATPγS to study the effects of eATP on root hair growth. Although Lew and Dearnaley (2000) found that a higher concentration ATP compared to ADP was needed to induce half maximal membrane depolarization in *Arabidopsis* root hairs (0.4 mM for ATP versus 10 μM for ADP), dose response curves showed that there was no difference between ADPβS or ATPγS on promotion or inhibition of growth. This may indicate that both ADP and ATP are recognized by the receptor that mediates root hair growth. On the other hand, AMPS and adenosine did not show similar effects on root hair growth, thereby indicating that the receptor does not recognize these smaller, albeit related molecules.

Application of ≥ 50 μM ODQ or ≥15 μM Ly, an NO antagonist, will inhibit growth. Meanwhile application of 50 or 75 μM NONOate, an NO donor, or 50 μM SNAP a NO agonist will promote root hair growth (Clark et al, 2010, in review). Moreover, co-incubation of 25 μM ODQ with 25 μM ADPβS blocked the promotion of growth in pre-washed root hairs. Additionally, Clark et al. (2010, in review) also studied the effects of eATP on the *nia1nia2* mutant, which have suppressed NO production. They found that the *nia1nia2* mutant had a lower average root hair growth rate compared to wild type. Moreover, the *nia1nia2* mutant

displayed no significant change in growth rate when treated with high concentrations of ATPγS or low concentrations of ATPγS with a wash. This indicates that NO plays a role downstream of eATP to affect root hair growth. Further studies may include other signaling agents downstream of eATP in the signal transduction pathway such as reactive oxygen species, ethylene, or auxin.

Appendix

Murashige and Skoog (MS) medium (100mL)
0.43 g MS salts (Sigma)
0.05 g MES
1 g sucrose
1 g agar (Sigma)
100 µL vitamin mixture

Vitamin Mixture Stock (1000x, 1 L)
1 g Nicotinic Acid
10 g Thiamine-HCl
1 g Pyridoxine-HCl
100 g Myo-Inositol

II. Transgenic Tomato Overexpressing *Arabidopsis* Annexin1

Abstract

Annexins are a multigene family of calcium-binding proteins that have been found in both plants and animals. In these cells, they play a role in diverse calcium-mediated signaling pathways. Recent studies have shown that certain plant annexins play a role in stress responses. For example, plants overexpressing annexin1 from *Arabidopsis thaliana* have greater resistance to biotic and abiotic stresses. Thus in this study, *Arabidopsis thaliana* annexin1 cDNA was transformed into tomato (*Solanum lycopersicum*, MicroTom) to test whether this annexin could confer stress tolerance in an agricultural crop. To do this, the annexin1 cDNA from *Arabidopsis* was amplified using polymerase chain reaction (PCR). This cDNA sequence was then inserted into several vectors to give it a promoter and correct directionality. The vector was transformed into *E. coli* to magnify the annexin1 vector to transfer it to *Agrobacterium* which was used to infect the tomato plant to transfer the annexin1 construct. The construct has been sent to the Jean Gould lab at Texas A&M for transformation into tomatoes. Discovering the effects of overexpressing annexin1 in transgenic tomatoes may allow agricultural use in locations which have been traditionally deemed as unacceptable for farm use. Additionally, success in tomatoes may lead to creation of other agricultural crops expressing enhanced levels of annexin1 for use in crop cultivation.

Introduction

Annexins bind cellular membranes in a calcium-dependent manner to perform a large variety of functions in the cell. Annexins have been found to play a role in membrane fusion and secretion relating to growth of a cell, as binding proteins, as well as serving directly as calcium channels. However, the role of annexin most significant to this study is that of stress resistance.

A number of studies have illustrated the response of annexins to biotic and abiotic stresses. Cantero et al. (2006) used quantitative real time reverse transcription PCR to find that the different *Arabidopsis* annexins are expressed at different levels when placed under various abiotic stresses. The four stresses tested were temperature modification to 4°C and 37ºC, dehydration, and increased salt concentration (250 mM NaCl) for two hours. The *Arabdiopsis* annexins were differentially regulated by these stresses, some upregulated and some downregulated. Each treatment produced a statistically significant change at the 95% confidence level for each of the eight different *Arabidopsis* annexins. This indicates that regulation of transcription of the annexin genes in *Arabidopsis* is changed in response to abiotic stresses.

Moreover, *Arabidopsis* annexin1 (AnnAt1) has also been found to play a role in reducing the reactive oxygen species (ROS) in a plant. Specifically, AnnAt1 has a 30-amino acid sequence that is homologous to heme-binding motifs in plant peroxidases (Clark and Roux, 1995; Gidrol et al., 1996). The peroxidase-like motif is located on the first repeat that is a part of the well-conserved type II Ca^{2+}-binding site. Because production of H_2O_2 (a ROS) is related to abiotic stresses, this peroxidase-like activity of annexin may confer increased resistance to abiotic stresses in plants. In particular, stress conditions will increase the production of ROS past the rate of breakdown due to the increased activity of enzymatic systems such as NADPH oxidase. The increased ROS levels will lead to subsequent oxidative damage to cells and cellular

membranes via peroxidation. ROS will also damage nucleic acids and proteins in the cell. Thus annexin may protect against the effects of ROS in plant cells.

Furthermore, Konopka-Postupolska et al. (2009) found that *Arabidopsis* plants overexpressing AnnAt1 showed lower levels of H_2O_2 accumulation by abscisic acid (ABA) treatment. On the other hand, knockout AnnAt1 plants showed higher levels of H_2O_2. To test the level of H_2O_2 in the epidermal peels, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA), an oxidation-sensitive fluorescent dye, was used. The result of this experiment indicates that AnnAt1 may play a role in neutralizing ROS and providing tolerance to H_2O_2 in a plant.

Konopka-Postupolska et al. (2009) also tested the drought resistance of AnnAt1 knockout and AnnAt1 overexpressing *Arabidopsis* plants. Plants were grown for four weeks in a growth chamber under short-day conditions. Next, the plants were put in a drought condition for two weeks. After five days, the AnnAt1 knockout plants began to wilt even as the control and overexpressing plants remained turgid and green. Additionally, red spots appeared on the abaxial sides of the knockout AnnAt1 leaves, thereby indicating phenylpropanoid accumulation, another symptom of drought. Moreover, even when the control plants began to wilt, the overexpressing plants were still turgid and green. Furthermore, when the plants were placed under drought conditions and then watered, the *Arabidopsis* plants overexpressing AnnAt1 were still able to flower and produce viable seeds. On the contrary, knock out AnnAt1 plants showed increased sensitivity to drought conditions.

As a forerunner to the current study, Jami et al. (2008) studied the ectopic expression of *Brassica juncea* annexin (AnnBj1) in transgenic tobacco plants (*Nicotiana tabacum*) under abiotic and biotic stress conditions. Abiotic stresses were mimicked using chemical manipulations – dehydration (mannitol), salt (NaCl), heavy metal ($CdCl_2$), and oxidative stress

(H_2O_2). Transgenic tobacco plants showed statistically significantly higher tolerance to these stresses as illustrated by the preservation of chlorophyll content of the treated leaves. This indicates that the AnnBj1 annexin may protect chlorophyll degradation against elevated levels of ROS. Moreover, when placed in stress conditions, seedlings overexpressing AnnAt1 generally grew larger and greener than the wild-type seedlings. The transgenic tobacco plants also showed increased resistance to biotic stress by the oomycete pathogen, *Phytophthora parasitica* var. *nicotianae*, as seen by decreased necrotized leaf area compared to wild-type. The transgenic plants also expressed significantly higher levels of PR-1, glucanase, chitinase, and osmotin proteins which may confer resistance against fungal pathogen infection. Thus, AnnBj1 may play a role in eliciting defense responses within the transgenic tobacco plant.

The rationale for this project is similar to that conducted by Jami et al. (2008). The purpose of this project was to overexpress *Arabidopsis thaliana* annexin1 (AnnAt1) gene in tomato (*Solanum lycopersicum*, cultivar MicroTom) to see if it will confer increased tolerance to both abiotic and biotic stresses. This is significant as this may be the first attempt to overexpress annexin in an agricultural crop. Providing stress tolerance to an important agricultural crop, such as tomato, may allow it to be grown in locations which have been traditionally deemed unsuitable for farm use due to high salt concentration in the soil or low water conditions. Additionally, if annexins can be transferred into an agricultural plant such as tomatoes, other experiments can be performed to create other stress resistant transgenic crops overexpressing annexin such as potato, corn or rice. The AnnAt1 gene was chosen for this study because several studies have illustrated its role in increased stress tolerance in plants. Additionally, AnnBj1 and AnnAt1 are highly similar with 91% homology (Jami et al., 2008); therefore, similar results regarding stress tolerance are expected with the transgenic tomatoes.

MicroTom tomatoes were selected as the model organism in studying tomatoes as they are small and dense with a shorter life cycle (70-90 days from seed to fruit ripening). Moreover, they can be transformed at frequencies up to 80% via *Agrobacterium* which makes it suitable for study in a laboratory. Additionally, MicroTom tomatoes only differ from the standard cultivars by two major genes, so that any genetic manipulations performed on MicroTom can be easily transferred to the standard tomato cultivar (Meissner et al, 1997).

Materials and Methods

Outline of Transformation Methods

Key among the basic steps of cloning a gene is that the target gene must be isolated from the bulk of the genome. To do this, PCR was performed on the cDNA library using annexin1 primers. The PCR product was then run through an agarose gel, and the correct band of DNA was cut out of the gel and isolated. The gene must then be inserted into a vector and transformed into bacteria. This was done several times in this project – TOPO TA/ TOPO 8 vector for sequencing; pRT100 vector for promoter region and poly-A tail; pCambia 2300 for insertion into tomato genome. Finally, the gene may be amplified in *E. coli*. Dr. Jean Gould's lab will subsequently transform the pCambia vector into *Agrobacterium* to transfer annexin1 into tomatoes. Then, the transformed tomatoes will return to the Roux lab for line selection and testing.

Tissue collection and RNA extraction

Leaf and flowers were collected from two week old *Arabidopsis thaliana* (Columbia strain) plants. Approximately 80-100mg of plant tissue was collected for each trial and stored in

sterile RNAse free Eppendorf tubes. Plant tissues were immediately frozen using liquid nitrogen after it was cut. Tissue breakdown and RNA extraction always immediately followed tissue collection. All materials used during RNA collection and extraction were autoclaved to be RNase free, as any contamination with RNase will degrade the RNA collected. Tissue was ground up using a small hand held mortar and pestle in liquid nitrogen.

First, the head of the pestle was sprayed with RNaseZAP (Ambion) and rinsed with tap water to remove traces of RNase. The head of the pestle was then dipped in liquid nitrogen to lower its temperature closer to that of the frozen plant tissue. Next, the pestle was attached to a battery-operated motor with the Eppendorf tube serving as a mortar. Plant tissue was ground for 10-20 min until the plant tissue appeared to be a fine powder. Finally, RNA extraction was performed using the Qiagen RNeasy Plant Minikit. RNA was eluted using DEPC water. Concentration of RNA was measured using the Nanodrop at the ICMB Core facility.

First-strand cDNA library

Reverse transcription was performed to create a first-strand cDNA library. First, a DNase reaction was performed to remove contaminant DNA already present in the RNA sample. This reaction mixture consisted of 1 μg RNA, 1 μL DNase buffer, 1 μL DNase (Invitrogen #18068-015) and enough DEPC water to a total volume of 10 μL. This reaction was run for 15 min at room temperature. The DNase was then inactivated with 1 μL 25mM EDTA at 65ºC for 10 min. To maintain constant temperature for this and subsequent steps, the procedures were performed using water baths. Second, 1 μL oligo dT (0.5 μg/ μL) and 1 μL dNTP (10 mM) was added to the solution at 65ºC. The oligo dT serves as a primer for the cDNA library. After 5 min, the mixture was put immediately on ice to halt the reaction. Third, 4 μL 5xRT buffer and 2

μL 0.1M DTT were added and incubated at 42°C for 2 min. Fourth, 1 μL Superscript RT III (Invitrogen) was added to elongate the cDNA strands via reverse transcription. This reaction was run at 50°C for 50 min. Finally, the entire mixture was placed in a 75°C bath for 15 min for inactivation.

Selecting AnnAt1 gene

Polymerase chain reaction was used to select and amplify the annexin1 gene from the cDNA library. Primers were ordered from Integrated DNA Technologies. To create the N' primer, the first 21 basepairs of AnnAt1 on 5' end was used. To create the C' primer, the reverse complement of the last 21 basepairs of AnnAt1 on the 3' end was used. Primers were diluted to a 20 μM working stock. Primer sequences were checked to ensure that they did not dimerize with each other to form primer dimers or with themselves to form hairpin structures.

TomTomAnnAt1N:
5' – ATG GCG ACT CTT AAG GTT TCT – 3'

TomTomAnnAt1C:
5' – TTA AGC ATC ATC TTC AAC GAG – 3'

The Roche Expand High Fidelity Plus PCR system was used, as it contains both *Taq* DNA polymerase for elongation in replication and a novel proofreading enzyme that does not have any polymerase activity. This reduces the error rate of the DNA strand produced. The following mixture was used for each PCR reaction.

Roche Expand High Fidelity PCR	
Reaction Buffer w/ $MgCl_2$	10 µL
dNTP (2.5 mM)	4 µL
Primer N' (20 µM)	5 µL
Primer C'	5 µL
cDNA library	2 µL
Enzyme mixture	0.5 µL
Water	23.5 µL
Total	50 µL

The thermal cycler instrument (Pelter Thermal Cycler PTC-200) was used to perform the PCR reactions. First, an initial denaturation step was performed by heating the reaction to 94°C for 2 min. This step was done to separate the DNA double helix to single stranded DNA. Second, the mixture was heated at 94°C for another 30s for the subsequent denaturation step in each repeated thermal cycle. Third, the mixture was annealed at 51°C for 30s. This step is used to anneal the primers onto the ends of the annexin1 DNA strand for amplification. Because only annexin1 primers were added, only the annexin1 gene within the cDNA library is amplified in the PCR reaction. Fourth, extension of the primers using *Taq* DNA polymerase was carried out at 72°C for 1 min and 15s. In this step, *Taq* DNA polymerase will add the appropriate dNTP to replicate the template strand of DNA. The proofreading enzyme will proofread the newly replicated strand and correct errors to maintain the fidelity of the PCR product. Then, steps two through four were repeated for another 35 cycles to increase the amount of annexin1 gene produced. Next, a terminal extension step was carried out at 72°C for 7 min to ensure that each DNA strand was completely replicated. Finally, the reaction was stored in the PCR machine at 4°C until ready for agarose gel electrophoresis. It is important to note that sometimes the completed mixture was transferred and stored in 4°C refrigerator if others needed to use the PCR machine.

In the initial testing phase of the *Arabidopsis* cDNA library, three controls were run to test if the primers were working correctly. These trials used the NEB Quickload Taq MasterMix for PCR. The Mastermix contains all components needed for PCR (*Taq* polymerase, buffer, dNTP) including the loading dye for subsequent agarose gel electrophoresis. The following table gives the composition of the control solutions.

Tube	C' primer	N' primer	cDNA	Water	Mastermix	Total
1	0.25 µL	–	1 µL	11.25 µL	12.5 µL	25 µL
2	–	0.25 µL	1 µL	11.25 µL	12.5 µL	25 µL
3	0.25 µL	0.25 µL	–	12.0 µL	12.5 µL	25 µL
4	0.25 µL	0.25 µL	1 µL	11.25 µL	12.5 µL	25 µL

Tube 1-3 should not produce any PCR product. Tube 1 contains only the C' primer and it is not sufficient to create the correctly sized annexin1 gene. Likewise, Tube 2 which contains only the N' primer cannot create the correctly sized annexin1 gene. Tube 3 does not contain any cDNA, therefore there is no template for the primers to anneal with. Only Tube 4 should produce PCR product as it contains both primers and cDNA.

Agarose gel electrophoresis was then used to identify and select the annexin1 DNA band. 100 mL of 1% agarose gel was created using the following procedure. First, 100mL TAE 1X (or 2mL TAE 50X with 98mL ddH_2O) was combined with 1 g of agarose and heated in microwave to melt the agarose. See appendix for TAE 50X recipe. Agarose is a carbohydrate that when heated is loose, but will form cross-linked repeated structures as it cools. These cross-linked structures will form pores which act as a sieve to separate DNA by size. Moreover, increasing the concentration of DNA within the agarose gel mixture will reduce the pore sizes to better separate DNA strands by size. The mixture was heated until no visible agarose granules could be seen. Then, 10 µL of 10,000X EtBr was added to the mixture and the gel solution was immediately poured into the gel plate. Ethidium bromide (EtBr) is added to the agarose gel to

help visualize DNA after electrophoresis. EtBr is an intercalating dye that will bind to the DNA by inserting itself into the double-stranded DNA. When excited with ultraviolet light at 254nm or 365nm, the pi-pi bonding interaction between the DNA and EtBr will shift emission into visible light at 600-650nm, resulting in orange light. Extreme caution must be used when handling EtBr as it is an extremely toxic mutagen. Any pipette tips that came in contact with EtBr was disposed of separately. Additionally, only certain flasks were used to create the agarose gel mixture to prevent contamination of EtBr to other glassware in the lab. Then the gel mixture was poured into a tray with combs to form wells. After the gel was cooled and set, 1X TAE tank buffer was added to each side of the apparatus until the gel was submerged. 10 μL 1kB ladder was loaded into well one, with PCR reactions loaded into subsequent lanes. If NEB Quickload Taq MasterMix was used during the PCR reaction, as was performed with the initial PCR reaction using controls, no dye was added. However, with the Roche Expand High Fidelity PCR system, 6X dye was added to PCR product at a 1:6 ratio before loading into the gel. Glycerol in the dye will increase density of the DNA product and prevent it from spilling out of the well. Additionally, the dye can be used to visualize the migration of smallest sized DNA within the gel. Approximately 20-25 μL of PCR product (including dye) was loaded into each well. With a 50 μL reaction, the PCR product was split into two wells to increase the amount of DNA to be collected in subsequent steps. Electrophoresis was run at constant voltage of 100V for approximately 30-45 min until the dye reached 1-2 cm from the end of the gel. Samples were run from negative to positive charge (black to red) as the phosphodiester backbone of DNA is negatively charged. Next, the gel was placed on a UV light box and photographed using AlphaImager 2000. The correct DNA bands containing the annexin1 PCR product was cut out of the gel and purified using Qiagen Qiaex II Gel Extraction Kit to produce 30 μL of product.

Sequencing AnnAt1

The PCR product obtained in the step above needed to be sequenced to confirm that it was indeed the desired annexin1 gene. Starting from this step, only annexin1 DNA obtained from *Arabidopsis* leaves was used. This was because the leaves produced a greater amount of DNA. To sequence the gene, the annexin1 PCR product was first cloned into the Invitrogen TOPO TA vector. This vector does not require restriction enzyme cutting or ligation. It works as the TOPO TA is a linearized vector with a single overhanging 3' T residue. On the other hand, *Taq* polymerase (from the Roche High-Fidelity Plus PCR system) is able to add a single A to the 3' end of a PCR product which can base pair with the overhanging 3' T to ligate into the vector. The following gives the setup of the TOPO TA cloning reaction. The TOPO TA vector map is included in the appendix.

TOPO TA cloning reaction	
PCR product	4 μL
Salt solution	1 μL
TopoTA vector	1 μL
Total	6 μL

This reaction was incubated at room temperature for 20 min to allow cloning to occur. Next, the TOPO TA vector was transformed into competent cells (Invitrogen OneShot Top10 Chemically Competent Cells). Chemically competent cells are usually *E. coli* cells that have been treated with a variety of chemicals to compromise the integrity of the cell membrane to make it more porous. The increased number of pores in the cell wall facilitates plasmid diffusion into the bacterial cell. In the first step of transformation, frozen competent cells (stored at -80ºC) and the cloning product were cooled on ice. Then 2 μL of the TOPO cloning reaction product was added to the vial of competent cells and gently mixed. After incubating the reaction on ice for 30 min, the cells were heat-shocked for 40s at 42ºC without shaking. The heat-shock step was used to

begin repair mechanisms of the cell wall. The solution was immediately transferred to ice for 2 min, then 300 µL of LB media warmed to 42ºC is added to the solution. Refer to the appendix for LB media and LB agar recipe. The bacterial solution was then placed horizontally in a shaking incubator at 200rpm and 37ºC for 1 hr to allow the transformed bacteria to replicate. The LB plates were placed in an incubator to prewarm them to 37ºC. Next, the solution was centrifuged and excess LB was poured off. Approximately 50µL of bacterial solution was plated on each LB agar plate and spread evenly across the surface of the agar. For TOPO TA cloning, LB agar plates containing 50µg/ mL carbenicillin were used. Bacterial plates were then placed in a 37ºC incubator for approximately 14 hrs, then moved to 4ºC cold room until use.

To screen the bacteria, eight colonies per plate were inoculated to a 5 mL liquid LB/ carbenicillin culture and grown overnight in a shaking incubator. Then, 1 mL of the culture was used for plasmid isolation using the Qiaprep Spin MiniPrep kit. Next, restriction digest using EcoRI (NEB) and agarose gel electrophoresis were performed. Correct DNA band size on the agarose gel (1 kb) determined whether or not the annexin1 gene was successfully cloned.

TOPO TA cloning was not successful. Four attempts were performed and sequences were of poor quality – complete sequences of the gene could not be obtained. Testing of the TOPO TA cloning kit using provided controls yielded no products. This indicated that the TOPO TA cloning kit may have been defective. Therefore, sequencing using the Invitrogen TOPO 8 cloning kit was used instead. TOPO 8 cloning mechanism and procedure are very similar to that of TOPO TA. The significance of TOPO 8 is that it can be used for recombinant Gateway cloning. However, that procedure is not needed in the current experimental design. However, TOPO 8 was used as an alternative to TOPO TA as other colleagues in the lab had successfully sequenced genes using the TOPO 8 system. The main change in experimental procedure for

TOPO 8 is that it only requires 5 min incubation for ligation to occur and uses spectinomycin as its selection gene. Please refer to the appendix for TOPO 8 vector map.

DNA product was sequenced by the ICMB core facilities. For TOPO TA and TOPO 8 cloning, M13 forward and reverse primers provided by the core facilities were used. Sequencing of the annexin1 gene in the TOPO 8 vector revealed that it did not contain the appropriate restriction sites to clone annexin1 into the pRT100 vector. Liquid bacterial culture containing the successful clones were stored as glycerol stocks at -80°C.

Transformation to pRT 100 vector

The annexin1 gene was transferred to the pRT100 vector to add the 35-S promoter to the N' end and a poly-A signal to the C' end of the gene. Therefore, inserting annexin1 into pRT100 must be directional to ensure that the gene is transcribed in the correct orientation. The CamV 35-S promoter (cauliflower mosaic virus) is the most commonly used constitutive promoter, which is ideal for the overexpression of annexin1 in tomato. However, the disadvantage of using the 35-S promoter is that it does not equally express the target protein throughout the plant. Decreased expression is found especially in pollen. Nonetheless, the 35-S promoter was used because overexpression in the fruit of the tomato plant was deemed most important. Additionally, pRT100 uses ampicillin as its selection gene. However, carbenicillin was used instead of ampicillin, because its byproducts are not as toxic as that of ampicillin.

To transfer the annexin1 gene from TOPO 8 to pRT100 vector, the appropriate restriction site needed to be added to the gene. Therefore, PCR using primers containing the restriction sites were used to amplify the annexin1 gene from TOPO 8 via PCR. Analysis of annexin1 gene revealed that of the restriction sites in the multiple cloning site, the following restriction enzymes

could be used: XhoI, ApaI, NcoI, SmaI, BamHI, and XbaI. Refer to the appendix for pRT100 vector map. To retain correct orientation of the annexin1 gene, XhoI restriction site was added to the N' primer and BamHI was added to the C' primer. These two enzymes were used instead of a combination of another two as they are both compatible with the same buffer system. The following displays the new primers used to add restriction sites to the annexin1 gene.

Ann1NpRTXhoI
5' – GCC G**C**|**T CGA G**AT GGC GAC TCT TAA GGT TTC T – 3'

Ann1CprTBamHI
5' – CTA G**G**|**G ATC C**TT AAG CAT CAT CTT CAC CGA G – 3'

PCR was performed using the Roche High Fidelity Plus PCR system to maintain correct sequence of the gene. Subsequent agarose gel electrophoresis, visualization on the UV light box, and gel purification were performed as described above. The expected size of the PCR product was 1kb. The resulting PCR product was annexin1 with restriction site and sticky ends added to both N' and C' ends.

The pRT100 vector was cut with restriction enzymes XhoI (NEB) and BamHI (NEB) to create sticky ends for ligation with the annexin1 PCR product. Restriction digest with pRT100 also used CIP, which removes the 5' phosphate group to prevent the vector from religating with itself. BSA was also added as an inactive protein to protect the restriction enzymes from degradation. The following gives the setup of restriction enzyme digest of pRT100.

pRT100 Restriction Digest	
pRT100 10X	5 µL
BamHI	2 µL
XhoI	2 µL
NEB Buffer 2	5 µL
BSA	0.5 µL
CIP	10 µL
ddH_2O	25.5 µL
Total	50 µL

Restriction digest was performed at 37ºC water bath for 2-3hrs. Then, agarose gel electrophoresis was performed to separate digested vector from undigested ones. Agarose gel purification was performed to obtain the digested vector.

With sticky ends on the vector and insert, ligation was performed. A 1:3 ratio of vector to insert was used to maximize the amount of ligated product in 8 µL. The following calculation was used to calculate the amount of vector and insert to add.

$$\frac{\dfrac{\text{Concentration of vector}}{\text{Size of vector}}}{\dfrac{\text{Concentration of insert}}{\text{Size of insert}}} = \frac{1}{3}$$

Concentration of the vector and insert were measured using the Nanodrop before hand.

The following gives the setup of a ligation reaction.

Ligation reaction	
DNA (vector + insert)	8 µL
Ligation buffer	1 µL
T4 DNA ligase (NEB)	1 µL
Total	10 µL

The reaction was incubated at 14ºC overnight using the thermal cycler machine used for PCR. Next, transformation to competent cells, screening via plasmid isolation, and agarose gel electrophoresis were performed as described above. Competent cells were plated on LB agar with 50µg/ mL carbenicillin. Restriction digest using PstI (NEB) was performed to cut out the new annexin1 gene product including the 35-S promoter and poly-A tail. The expected band size for the gene product was 1.5kb. Undigested plasmid DNA, AnnPRT was saved as it contains the target product. Liquid bacterial culture containing the successful clones were stored as glycerol stocks at -80°C.

Transformation to pCambia 2300 vector

The annexin1 gene was transferred to the pCambia vector for transformation into *Agrobacterium* and tomato plants. pCambia vectors are ideal for transformation into plants as they have a high copy number in *E. coli* for high DNA yields and are highly stable in *Agrobacterium.* Moreover, the pCambia 2300 is a small plasmid, 8642bp, which means it will be easily taken up by bacteria. pCambia 2300 is a minimal selection vector, therefore it does not contain promoter and terminator sequences by itself. Additionally, it uses kanamycin for both plant and bacteria selection. Because promoter sequence, AnnAt1 DNA sequence and poly-A tail are inserted, the orientation of the insert does not matter. pCambia 2300 uses the pUC18 polylinker restriction map. Please refer to the appendix for pCambia 2300 vector map and pUC18 polylinker restriction map.

To transfer the annexin1 gene from pRT100 vector to pCambia, the restriction site outside the 35-S promoter and poly-A tail were used. Restriction enzymes on pRT100 that could be used were HindIII, SphI, and PstI. However, HindIII will cut within the annexin1 gene and no SphI restriction site is not present on the pUC18 polylinker, therefore PstI was used. Next, both pCambia 2300 vector and pRT100 vector containing annexin1 were cut using PstI using the following setup.

pCambia 2300 Restriction Digest

pCambia 2300 DNA	10 µL
PstI	4 µL
NEB Buffer 3	5 µL
BSA	0.5 µL
CIP	1 µL
ddH_2O	29.5 µL
Total	50 µL

AnnPRT Restriction Digest	
AnnPRT DNA	15 µL
PstI	4 µL
NEB Buffer 3	5 µL
BSA	0.5 µL
ddH_2O	25.5 µL
Total	50 µL

CIP was added only to the pCambia vector to prevent it from religating with itself. If CIP was added to both insert and vector, religation could not occur without the extra phosphate. Restriction digest was performed at 37ºC water bath for approximately 3hrs. Then, agarose gel electrophoresis was performed to separate digested vector from undigested ones. Agarose gel purification was performed to obtain the digested vector and annexin1 product. Next, ligation of AnnPRT and pCambia 2300, transformation to E. coli, and screening were performed as described above. Competent cells were plated on LB agar with 50µg/ mL kanamycin. Finally, several of the successful clones, AnnpCam, were sent to the ICMB core facilities for sequencing. Liquid bacterial culture containing the successful clones were stored as glycerol stocks at -80°C.

Collaboration with Dr. Jean Gould

Because Dr. Roux's laboratory does not have extensive experience with transformation of tomatoes, AnnpCam was sent to Dr. Jean Gould's laboratory at the University of Texas A&M. The Gould lab will transform the AnnpCam DNA product into *Agrobacterium* and transfect tomato plants. *Agrobacterium* is used to transfect the tomato plants as it can horizontally transfer the target vector into the tomato plant and cause tumors. The Gould lab will incubate tomato cotyledons with *Agrobacterium* to create plant tissue cultures. These tissue cultures can then be used to regenerate whole plants. Transformed tomato plants will be sent back to the Roux lab for line selection and testing of tolerance to abiotic and biotic stresses.

Further Testing of Tomatoes

A group of wild-type tomatoes were grown in soil for seed collection and future testing as a control. MicroTom seeds were purchased from the Tomato Growers Supply Company. Seeds were sterilized using the procedure as described by Lima et al. (2004). The tomato seeds were surface sterilized in 50% commercial bleach and 0.1% Tween-20 for 20 min. Next, the seeds were rinsed six times with autoclaved distilled water. Prior to planting, seeds were vernalized in the dark at 4°C for 4 days. Soil (Sungro Metro-Mix 2000) was autoclaved prior to use. The sterilized seeds were planted approximately 1.5cm under lightly packed damp soil in 325 mL flower pots. Soil was kept moist by providing about 1-1.5 cm of water in a large bin containing the flower pots. The bins were placed on a heated surface between 20-23°C under 24hr light conditions. When the tomato plant reached about 20 cm high, stake and twine was used to help it remain upright.

Tomato seed collection

Ripe tomato fruit was collected and squeezed to extract the seeds. Excess fruit and the plant tissue was scraped off and discarded. The seeds were then rinsed with distilled water. Seeds were then placed in an Erlenmeyer flask covered with cellophane and soaked in approximately 3cm distilled water. This was done to remove the seed coat of the seed which prevents germination. After 3 days, excess water containing the seed coat was poured off and the seeds were transferred to a paper towel to dry.

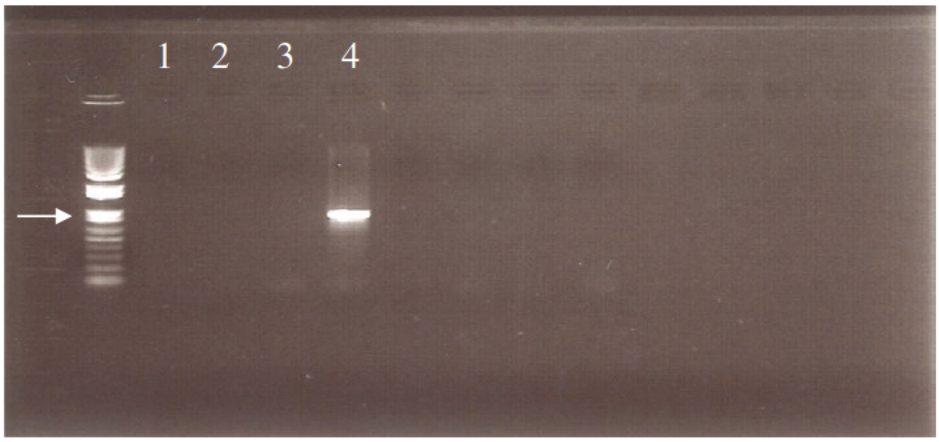
Testing tomato annexins

Tomato tissue culture was collected for a qualitative screening of the presence of tomato annexin p34 in different tissues of the plant. Moreover, the tissue culture was tested using the AnnAt1 primers to ensure that there is no noise from the primers. Therefore, in the future transgenic tomatoes may be screened using these primers. The following tissues were collected from the MicroTom tomatoes – leaf, flower, red skin, ripe fruit (red), and raw fruit (green). RNA collection and first-strand cDNA was performed as described above. PCR was performed with the two sets of primers using the NEB Quickload Taq MasterMix system with a 50ºC annealing temperature.

Results

Initial testing of the *Arabidopsis* leaf cDNA library revealed AnnAt1 only under expected conditions. Lanes 1-3 contained control solutions that did not result in DNA product as they did not contain all materials required for replication. Lane 4 gives a positive result with a correct band size of 1kb, indicating that it is AnnAt1.

Figure 1. Control screening of cDNA library



After successful cloning into TOPO 8, resulting plasmids were sent to the ICMB core facility for sequencing. The following gives the annexin1 sequence obtained through TOPO 8 cloning. M13 forward and reverse primers supplied by the ICMB core facility were used. Forward and reverse sequences translated to protein are presented together below.

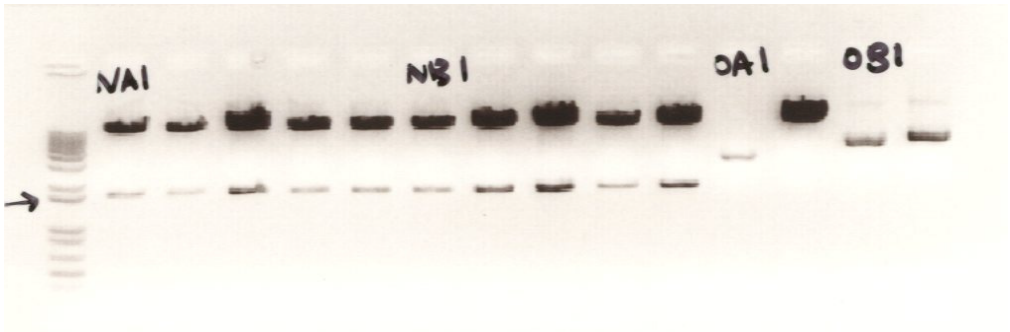
MATLKVSDSVPAPSDDAEQLRTAFEGWGTNEDLIISILAHRSAEQRKVIRQAYHETYGED
LLKTLDKELSNDFERAILLWTLEPGERDALLANEATKRWTSSNQVLMEVACTRTSTQL**P**H
ARQAYHARYKKSLEEDVAHHTTGDFRKLLVSLVTSYRYEGDEVNMTLAKQEAKLVHEKIK
DKHYNDEDVIRILSTRSKAQINATFNRYQDDHGEEILKSLEEGDDDDKFLALLRSTIQCL
TRPELYFVDVLRSAINKTGTDEGALTRIVTTRAEIDLKVIGEEYQRRNSIPLEKAITKDT
RGDYEKMLVALLGEDDA **Stop**

Leu[119] has mutated into Proline. This sequence did not contain the appropriate restriction sites for cloning into the pRT100 vector. Therefore, as mentioned new primers were created to add restriction sites to the ends of the AnnAt1 gene.

AnnAt1 successfully cloned into PCambia 2300

The AnnAt1 with 35-S promoter and poly-A tail from pRT100 was cloned into pCambia 2300. This figure illustrates successful cloning as 1.5kb bands can be seen in the first 10 lanes. Concentration of plasmid DNA of the successful clones was measured using Nanodrop and samples NA3, NB3, NB2 were sent to the Gould lab for transformation to *Agrobacterium*. The first 10 samples came from a ligation reaction using freshly restriction digested insert, while the final four samples came from an older sample of insert. The older samples did not produce successful cloning as seen by the incorrect band size.

Figure 2. Screening of bacteria transformed with AnnpCam



The following gives the final DNA sequence of AnnAt1 in the pCambia 2300 vector. Sequencing was performed using M13 forward and reverse primers as provided by ICMB core. Sequencing also revealed that the AnnAt1 construct with 35-S promoter and poly-A tail were inserted into the pCambia 2300 vector in reverse sequence. However, this does not affect the efficiency of the construct.

MATLKVSDSVPAPSDDAEQLRTAFEGWGTNEDLIISILAHRSAEQRKVIRQAYHETYGED
LLKTLDKELSNDFERAILLWTLEPGERDALLANEATKRWTSSNQVLMEVACTRTSTQL**P**H
ARQAYHARYKKSLEEDVAHHTTGDFRKLLVSLVTSYRYEGDEVNMTLAKQEAKLVHEKIK
DKHYNDEDVIRILSTRSKAQINATFNRYQDDHGEEILKSLEEGDDDDKFLALLRSTIQCL
TRPELYFVDVLRSAINKTGTDEGALTRIVTTRAEIDLKVIGEEYQRRNSIPLEKAITKDT
RGDYEKMLVALLGEDDA **Stop**

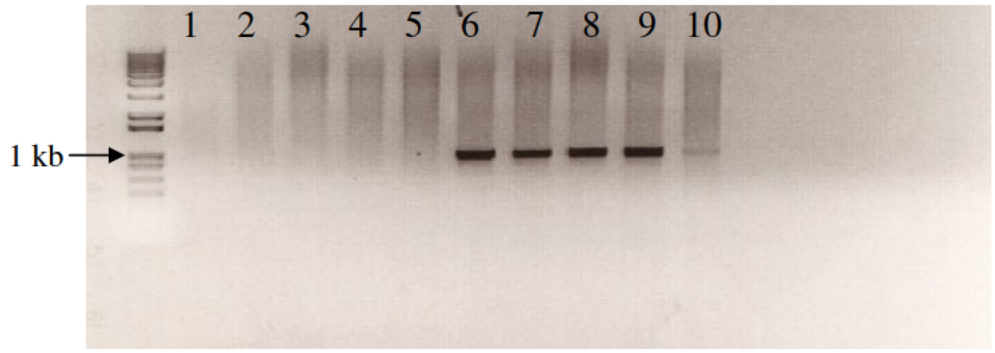
Sequencing of the final AnnAt1 sequence also contained the point mutation of Leu^{119} to Pro.

Tomato annexin p34 exists in most tomato tissues

RNA extraction, first-strand cDNA, and PCR were performed on various tomato tissues. Figure 3 gives the gel electrophoresis results of the PCR testing tomato annexins. Lanes 1-5 used the AnnAt1 primers, and produced no bands. This is a positive result as no *Arabidopsis* annexin should be expected in wild-type tomatoes. Moreover, this indicates that the AnnAt1 primers may be used for line selection of the transgenic tomatoes which will overexpress AnnAt1. Lanes 6-10 used the tomato annexin p34 primers. Samples were loaded in this order – ripe fruit, skin

(red), flower, raw fruit (green), and leaf. The clear band present in each lane at approximately 1kb indicates that tomato annexin p34 is expressed in all of the plant tissues. Although it appears that the tomato leaf expresses the least amount of annexin p34, this may not be true as loading controls and annexin specific primers were not used. Thus, the results of this experiment can only be interpreted qualitatively, rather than quantitatively. To quantitatively evaluate the concentration of annexin p34 in each tomato tissue, a separate loading control primer must be run. Additionally, gene specific primers must be used.

Figure 3. Screening of AnnAt1 primers and tomato annexin p34 primers



Discussion

In this study, the full-length cDNA for AnnAt1 was isolated and cloned into the pCambia 2300 vector for transformation to *Solanum lycopersicum*, cultivar MicroTom. The constitutive CamV 35-S promoter and poly-A tail were added using the pRT100 vector. A point mutation was detected of Leu^{119} to Pro. This point mutation may not be critical as it does not appear to be highly conserved in comparison to tomato annexin p34 and p35.

To date, the Gould lab has successfully transformed the AnnpCam vector into *Agrobacterium*; the next step will be to infect wild-type MicroTom tomato cotyledons by incubating them with *Agrobacterium* suspended in Virulence Induction Medium. The infected

cotyledons will then be grown on medium containing kanamycin (the selection antibiotic) with shoots appearing within 4-6 weeks. Finally, the shoots can be grown in rooting media with kanamycin and antibiotics to prevent *Agrobacterium* growth. When the seedlings are large enough they can be transplanted to soil. The second generation of MicroTom tomatoes can be used for line screening and testing.

When the lines of MicroTom overexpressing AnnAt1 have been identified, stress response tests similar to those used by Jami et al. (2008) will be performed. Chemicals can be used to induce stress treatments to mimic natural abiotic conditions to see if AnnAt1 does confer tolerance to stress. Mannitol will be used to induce the dehydration stress response, whereas NaCl and CdCl will be used to study the effects of high salt or heavy metal concentrations on the tomato plant. H_2O_2 as a representative reactive oxidative species will be used to study the effects of oxidative stress. To study stress effects in the seedling stage, root and leaf growth will be assayed after several weeks of growth. In the adult tomato plants, leaf disks will be assayed for chlorophyll levels. Moreover, if AnnAt1 does confer stress tolerance to adult tomato plants, the stressed plants must be able to produce flowers and viable seeds. Finally, the tomato fruit size and color during stress response will be examined as it is the part of the plant that is agriculturally important.

Takahashi et al. (2005) cataloged the response of MicroTom tomatoes to common fungal, bacterial, and viral pathogens. They found that the fungal diseases *Athelia rolfsii*, *Botryotinia fuckeliana*, *Oidium sp., Phytophthora infestans*, and *Sclerotinia sclerotiorum* caused necrotic lesions that quickly spread across the plant leading to wilting and death. However, MicroTom appeared to be resistant to *Alternaria alternata*, *Corynespora cassiicola*, and *Fusarium oxysporum*. Although some tiny lesions appeared, which may indicate hypersensitive cell death,

the lesions did not spread and the plant remained alive and strong. Additionally, five strains of the bacteria *Ralstonia solanacearum* (MAFF301526, MAFF730103, MAFF301522, MAFF730138, and OE1-1), induced wilting in the plant within 16 days after inoculation. On the other hand, when inoculated with *Pseudomonas syringae pv. tomato, P. s. pv. tabaci,* or *P. s. pv. glycinea*, bacteria populations did not increase, causing only yellow lesions on leaves. Finally, *Tomato mosaic virus, Tomato aspermy virus,* and *Cucumber mosaic virus* caused severe systematic necrosis leading to death of the plant. Additionally *Tomato aspermy virus* produced seedless fruit. To study the effects of biotic stresses of fungal, bacterial and viral pathogens, similar tests as those performed by Takahashi et al. (2005) can be used. Moreover, their results may serve as a guide or comparison. If AnnAt1 does confer biotic stress tolerance to the transformed tomatoes, severe systematic necrosis and death should not be observed in response to fungal, bacterial, and viral pathogens.

This experiment has broad implications. If the tomato plant, an important agricultural crop, can be given higher stress tolerance, it can be grown in locations which have been traditionally deemed as unsuitable for agricultural use due to poor soil conditions or low water conditions. This will allow more areas which can be used for agriculture, creating more jobs and food for the populace. Moreover, if the AnnAt1 gene can be transferred into an agricultural plant such as tomatoes, other experiments can be performed to create other transgenic crops such as potato, corn or rice.

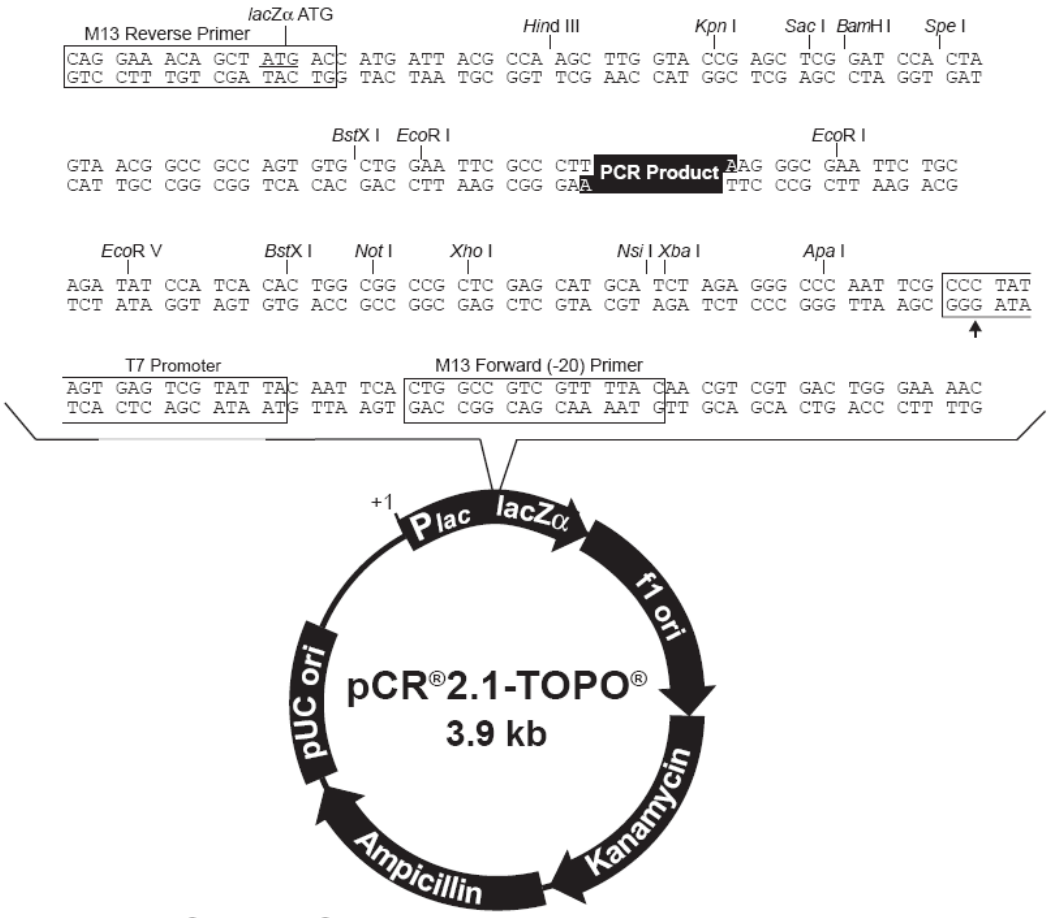
Appendix

TAE 50X

Tris base	242 g
Glacial Acetic Acid	57.1 mL
EDTA	18.6 g

Adjust volume to 1 L with distilled H_2O.

Figure 4. Invitrogen TOPO TA vector map



LB liquid media

Tryptone	2 g
Yeast Extract	1 g
NaCl	2 g

Adjust volume to 200 mL with distilled H_2O. Solution must be autoclaved before use. Antibiotic solutions were added just prior to use.

LB agar

Tryptone	2 g
Yeast Extract	1 g
NaCl	2 g
Agar	3 g

Adjust volume to 200 mL with distilled H_2O. Solution must be autoclaved before use. Antibiotic solutions were added when the agar was slightly cooled and immediately poured to plates.

Figure 5. Invitrogen TOPO 8 vector map

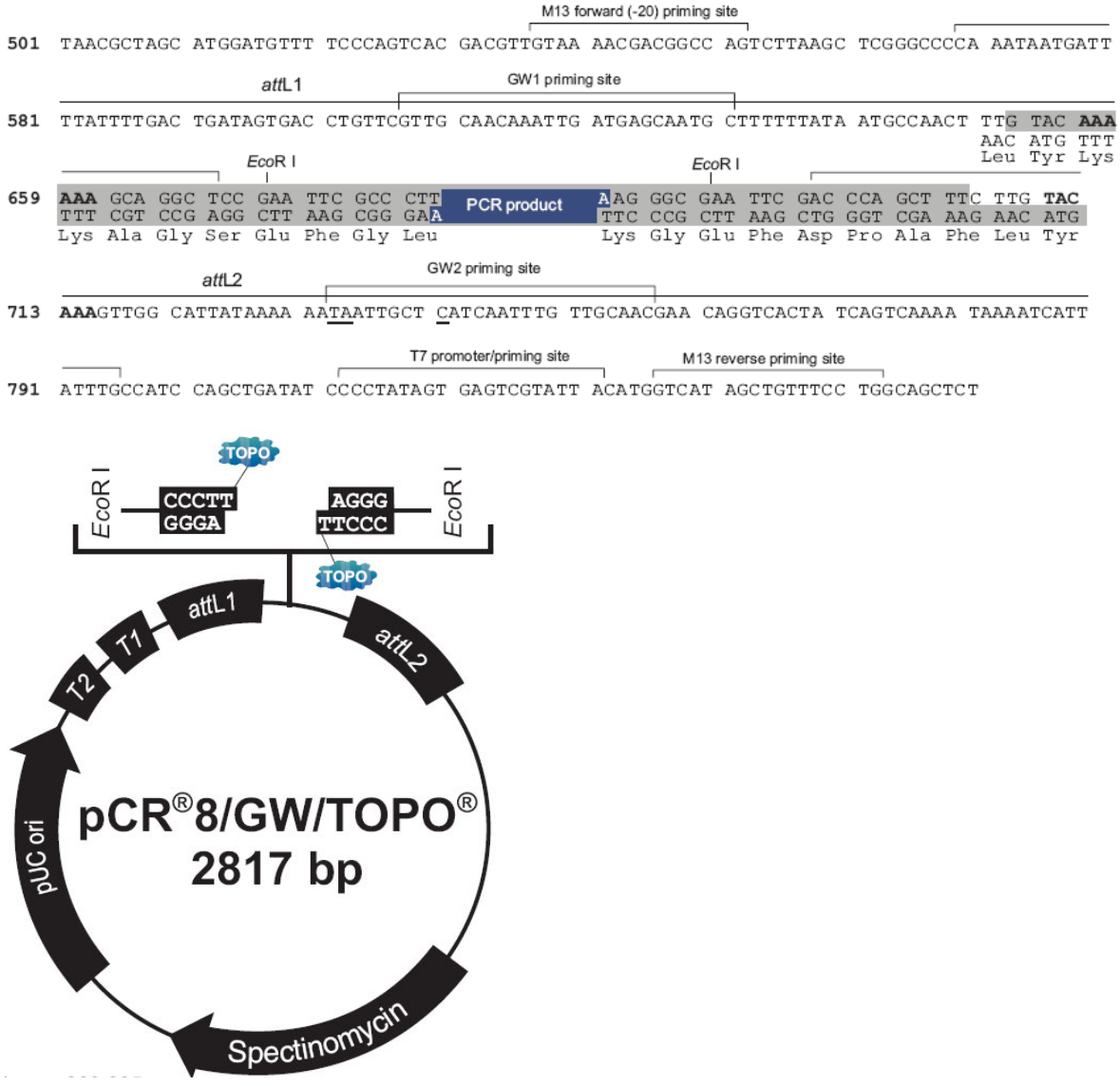


Figure 6. pRT100 vector map

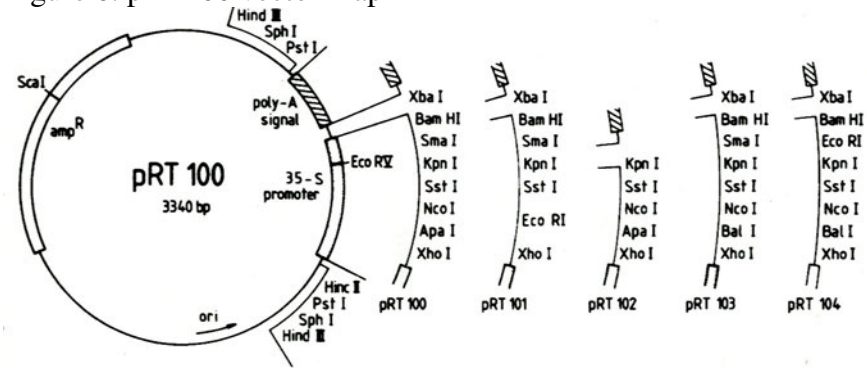


Figure 7. pCambia 2300 vector map

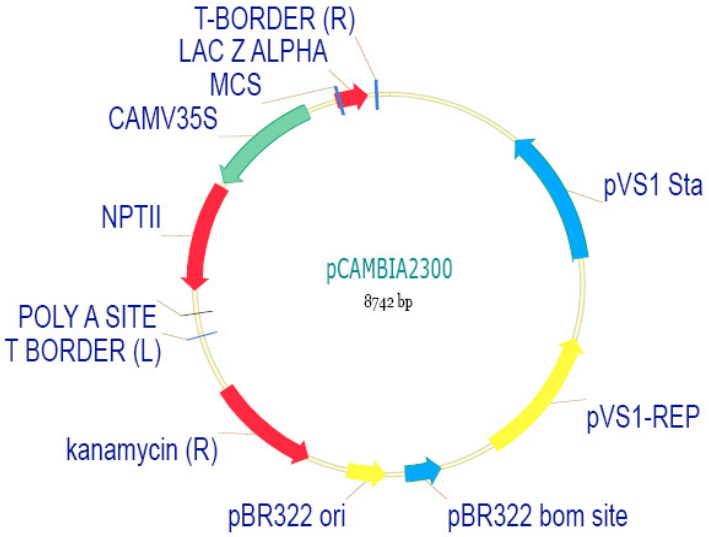
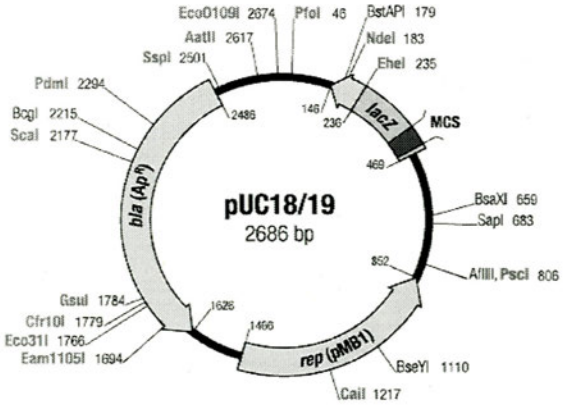


Figure 8. pUC 18 multiple cloning sites



Multiple Cloning Sites

AnnAt1 homology in tomato

The entire genome of *Arabidopsis thaliana* has already been sequenced, and the annexin1 sequence was obtained by performing a BLAST search. The following gives the reported annexin1 cDNA sequence and protein sequence.

Arabidopsis annexin1 cDNA sequence, *AnnAt1*:

ATGGCGACTCTTAAGGTTTCTGATTCTGTTCCTGCTCCTTCTGATGATGCTGAGCAATTGAGAAC
CGCTTTTGAAGGATGGGGTACGAACGAGGACTTGATCATATCAATCTTGGCTCACAGAAGTGCTG
AACAGAGGAAAGTCATCAGGCAAGCATACCACGAAACCTACGGCGAAGACCTTCTCAAGACTCTT
GACAAGGAGCTCTCTAACGATTTCGAGAGAGCTATCTTGTTGTGGACTCTTGAACCCGGTGAGCG
TGATGCTTTATTGGCTAATGAAGCTACAAAAAGATGGACTTCAAGCAACCAAGTTCTTATGGAAG
TTGCTTGCACAAGGACATCAACGCAGCTGCTTCACGCTAGGCAAGCTTACCATGCTCGCTACAAG
AAGTCTCTTGAAGAGGACGTTGCTCACCACACTACCGGTGACTTCAGAAAGCTTTTGGTTTCTCT
TGTTACCTCATACAGGTACGAAGGAGATGAAGTGAACATGACATTGGCTAAGCAAGAAGCTAAGC
TGGTCCATGAGAAAATCAAGGACAAGCACTACAATGATGAGGATGTTATTAGAATCTTGTCCACA
AGAAGCAAAGCTCAGATCAATGCTACTTTTAACCGTTACCAAGATGATCATGGCGAGGAAATTCT
CAAGAGTCTTGAGGAAGGAGATGATGATGACAAGTTCCTTGCACTTTTGAGGTCAACCATTCAGT
GCTTGACAAGACCAGAGCTTTACTTTGTCGATGTTCTTCGTTCAGCAATCAACAAAACTGGAACT
GATGAAGGAGCACTCACTAGAATTGTGACCACAAGAGCTGAGATTGACTTGAAGGTCATTGGAGA
GGAGTACCAGCGCAGGAACAGCATTCCTTTGGAGAAAGCTATTACCAAAGACACTCGTGGAGATT
ACGAGAAGATGCTCGTCGCACTTCTCGGTGAAGATGATGCTTAA

Arabidopsis annexin1 protein sequence, *AnnAt1*:

MATLKVSDSVPAPSDDAEQLRTAFEGWGTNEDLIISILAHRSAEQRKVIRQAYHETYGEDLLKTL
DKELSNDFERAILLWTLEPGERDALLANEATKRWTSSNQVLMEVACTRTSTQLLHARQAYHARYK
KSLEEDVAHHTTGDFRKLLVSLVTSYRYEGDEVNMTLAKQEAKLVHEKIKDKHYNDEDVIRILST
RSKAQINATFNRYQDDHGEEILKSLEEGDDDDKFLALLRSTIQCLTRPELYFVDVLRSAINKTGT
DEGALTRIVTTRAEIDLKVIGEEYQRRNSIPLEKAITKDTRGDYEKMLVALLGEDDA

Using the AnnAt1 protein sequence, BLAST was performed to find homologous tomato annexin 1 sequences. Two matches were found tomato annexin p34 and p35 as shown below.

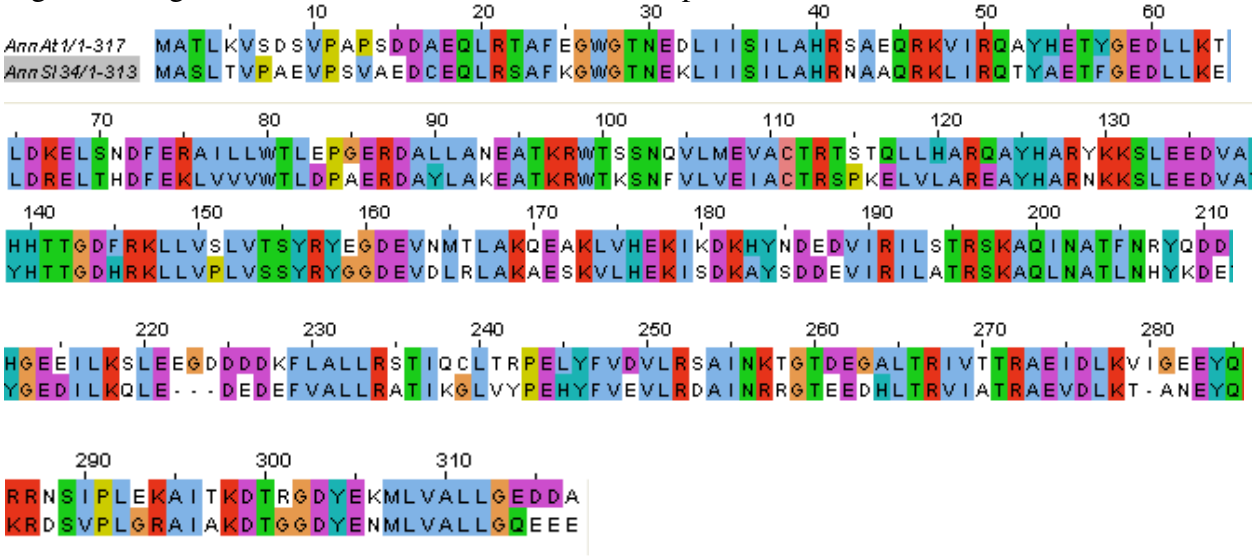
Tomato Annexin p34 cDNA sequence:

```
   1 atggcaagtc ttacagttcc ggcagaagtt ccttcagtcg ctgaagactg tgaacaactc
  61 cgatctgcct tcaaaggatg gggaacgaat gagaagttga ttatatcaat tttggctcat
 121 agaaatgcgg ctcaacgcaa attgattcga cagacttatg ctgagacttt tggggaagat
 181 ctgcttaaag agttggacag agaacttact catgattttg agaaattggt ggtagtatgg
 241 acactggatc ctgcagaacg tgatgcctat ttggctaagg aagctactaa gagatggaca
 301 aaaagcaact ttgttcttgt ggagatagct tgtaccagat ctcctaaaga actggttttg
 361 gcaagagaag cttatcatgc tcgtaacaag aaatctctcg aagaggacgt tgcttatcac
 421 actactgggg atcaccgcaa gcttttggta cctcttgtga gctcctaccg atatggggga
 481 gatgaggtgg acttgcgact tgctaaagca gaatctaaag tgctgcatga gaagatctcc
 541 gataaggctt acagtgacga tgaggtcatt agaattttag ccacaaggag caaagcgcaa
 601 ctcaatgcta ctttgaatca ttacaaagat gaatatggtg aggatatcct aaagcaatta
 661 gaagatgagg atgagtttgt tgcactgtta agggccacca taaaaggtct tgtctacccc
 721 gagcactatt tcgtggaggt tcttcgtgat gcaattaaca ggagaggaac agaggaagat
 781 catctaaccc gagttatcgc tacaagggct gaggtcgatc tgaagactat cgctaacgag
 841 taccagaaga gggatagcgt tcctctgggt cgcgccattg ccaaagatac aggaggagat
 901 tatgagaata tgctggtggc tttacttgga caagaggagg aataagaagc ggattggctc
 961 acttctgttt ataatgacca gataatatgc cattctccat atatttcaga gttggcatgt
1021 gtttgatgat tgagagtggt ctgttcacat gagctttagt ccttttcttc ttgtgagaaa
1081 ctttgaatat gaatctttgt gctgtctaaa aatgttctct aatgatttgc atccactaaa
1141 aaaaaaaaaa aaaaaaaaaa
```

Tomato Annexin p34 protein sequence:

```
MASLTVPAEVPSVAEDCEQLRSAFKGWGTNEKLIISILAHRNAAQRKLIRQTYAETFGEDLLK
ELDRELTHDFEKLVVVWTLDPAERDAYLAKEATKRWTKSNFVLVEIACTRSPKELVLAREAYH
ARNKKSLEEDVAYHTTGDHRKLLVPLVSSYRYGGDEVDLRLAKAESKVLHEKISDKAYSDDEV
IRILATRSKAQLNATLNHYKDEYGEDILKQLEDEDEFVALLRATIKGLVYPEHYFVEVLRDAI
NRRGTEEDHLTRVIATRAEVDLKTANEYQKRDSVPLGRAIAKDTGGDYENMLVALLGQEEE
```

Figure 9. Alignment of AnnAt1and Tomato annexin p34



BLAST e-value is $8x10^{-122}$ with a score of 429.

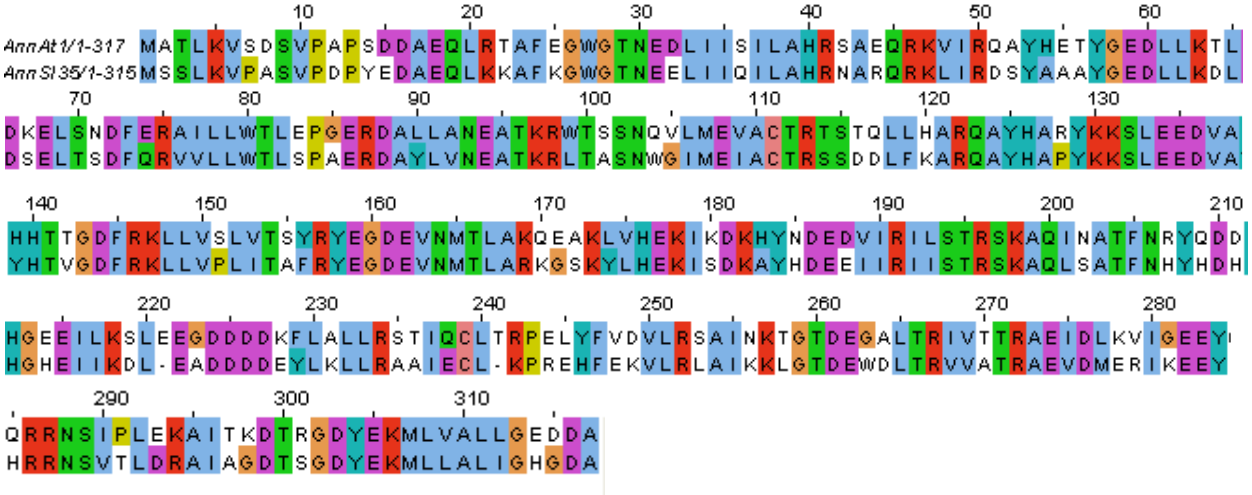
Tomato Annexin p35 cDNA sequence:

```
   1 atgtctagtc ttaaagttcc agcatcagtt ccagatcctt atgaagatgc tgagcaactc
  61 aaaaaagctt ttaaaggatg gggcacaaat gaggaactta ttattcagat tctggctcat
 121 aggaatgcca gacaacgcaa gttaatccga gattcttatg ctgctgctta tggagaggat
 181 cttctcaagg acttggattc tgaactgaca agtgattttc agcgtgtggt gcttctctgg
 241 actttgagtc ctgctgagcg cgacgcctac ttggttaatg aggctaccaa acgtctgact
 301 gctagcaatt ggggtatcat ggaaattgct tgtaccaggt cttctgatga tctttttaag
 361 gcgaggcagg cctaccatgc tccatacaag aaatcacttg aagaagatgt tgcttatcat
 421 acagtggggg atttccgtaa gcttttggtt cctcttataa ctgcattcag atatgaagga
 481 gatgaggtga acatgacatt agcaagaaag ggaagcaaat atctgcatga gaagatctct
 541 gacaaggctt accatgacga ggagatcatc cgaatcattt ctactaggag taaagcacag
 601 ctgagtgcta cgttcaacca ctaccatgat caccatggcc atgaaatcat caaggatctg
 661 gaagctgatg atgacgatga gtacctgaaa ctactcagag cagcaataga atgcttgaaa
 721 cccagagaac actttgagaa agttcttcga ttggctatca agaagctggg tacagacgaa
 781 tgggatctta ctagagttgt tgccactcgg gctgaagttg acatggagcg tatcaaagaa
 841 gagtaccata ggaggaacag tgttacattg gaccgtgcaa ttgctggaga cacttcagga
 901 gactatgaaa aaatgcttct ggctctgatt gggcacggag atgcttgaat tacatgtgct
 961 gaaaccttaa gataataaaa aactctactt attttctgaa ctttcatttg cttttatgat
1021 ctatggtgtg tactctcaga gtttggttct gtgtttatat gaactaaaaa cactcgggag
1081 ttgagttgtg ttttgttttc gccttcactt ttcatttcgg acttctactg gttttgcctg
1141 ctaaataagc atagcttcaa ctttggcttg aacggatctt gtttctttat aactcagaaa
1201 tagattatgt atcttggttc gtaaaaaaaa aaaaaaaaaa aa
```

Tomato Annexin p35 protein sequence:

```
MSSLKVPASVPDPYEDAEQLKKAFKGWGTNEELIIQILAHRNARQRKLIRDSYAAAYGEDLLKD
LDSELTSDFQRVVLLWTLSPAERDAYLVNEATKRLTASNWGIMEIACTRSSDDLFKARQAYHAP
YKKSLEEDVAYHTVGDFRKLLVPLITAFRYEGDEVNMTLARKGSKYLHEKISDKAYHDEEIIRI
ISTRSKAQLSATFNHYHDHHGHEIIKDLEADDDDEYLKLLRAAIECLKPREHFEKVLRLAIKKL
GTDEWDLTRVVATRAEVDMERIKEEYHRRNSVTLDRAIAGDTSGDYEKMLLALIGHGDA
```

Figure 9. Alignment of AnnAt1and Tomato annexin p35



BLAST e-value is $8x10^{-124}$ with a score of 436.

The *Arabidopsis* and tomato annexins p34 and p35 are fairly closely related. Tomato annexin p35 is slightly more homologous with a lower e-value, chance that the homology is due to chance only, and a higher BLAST score.

III. References

Cantero A, Barthakur S, Bushart T, Morgan RO, Fernandez MP, Chou S, Clark G, Roux SJ. 2006. Expression profiling of the Arabidopsis annexin gene family during abiotic stress, germination and de-etiolation. *Plant Physiology and Biochemistry* **44**: 13–24.

Clark GB, Roux SJ. 1995. Annexins of plant cells. *Plant Physiology* **109**: 1133-1139.

Clark G, Wu M, Wat N, Onyirimba J, Pham T, Herz N, Ogoti J, Gomez D, Canales A, Aranda G, Blizard M, Nyberg T, Terry A, Torres J, Wu J, Roux SJ. 2010. Genetic evidence for the roles of nitric oxide and reactive oxygen species in regulating both the stimulation and inhibition of root hair growth induced by extracellular nucleotides in *Arabidopsis*. *Plant Molecular Biology.* In review.

Demidchik V, Nichols C, Oliynyk M, Dark A, Glover BJ, Davies JM. 2003. Is ATP a signaling agent in plants? *Plant Physiology* **133**: 456-461.

Foresi NP, Laxalt AM, Tono´n CV, Casalongue´ CA, Lamattina L. 2007. Extracellular ATP induces nitric oxide production in tomato cell suspensions. *Plant Physiology* **145**:589–592.

Gidrol X, Sabelli AK PA, Fern YS, Kush. 1996. Annexin-like protein from *Arabidopsis thaliana* rescues delta *oxyR* mutant of *Escherichia coli* from H_2O_2 stress. *Proceedings of National Academy of Sciences U.S.A.* **93**: 11268-11273.

Jami SK, Clark GB, Turlapati SA, Handley C, Roux SJ, Kirti PB. 2008. Ectopic expression of an annexin from *Brassica juncea* confers tolerance to abiotic and biotic stress treatments in transgenic tobacco. *Plant Physiology and Biochemistry*. **46**:1019-1030.

Jeter CR, Tang W, Henaff E, Butterfield T, Roux SJ. 2004. Evidence of a novel cell signaling role for extracellular adenosine triphosphates and diphosphates in *Arabidopsis*. *Plant Cell* **16**:2652-2664.

Kim SY, Sivaguru M, Stacey G. 2006. Extracellular ATP in plants. Visualization, localization, and analysis of physiological significance in growth and signaling. *Plant Physiology* **142**:984-992

Konopka-Postupolska D, Clark G, Goch G, Dębski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J. 2009. The role of annexin 1 in drought stress in *Arabidopsis*. *Plant Physiology* **150**: 1394-410.

Lew RR, Dearnaley JDW. 2000. Extracellular nucleotide effects on the electrical properties of growing *Arabidopsis thaliana* root hairs. *Plant Science* **153**:1-6.

Lima JE, Carvalho RF, Neto AT, Figueira A, Peres LEP. 2004. Micro-MsK: a tomato genotype with miniature size, short life cycle, and improved in vitro shoot regeneration. *Plant Science* **167**: 753-757.

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy A. 1997. A new model system for tomato genetics. *The Plant Journal* **12**: 1465-1472.

Reichler SA, Torres J, Rivera AL, Cintolesi VA, Clark G, Roux SJ. 2009. Intersection of two signaling pathways: Extracellular nucleotides regulate pollen germination and pollen tube growth via nitric oxide. *Journal of Experimental Botany* **60**:2129-2138

Takahashi H, Shimizu A, Arie T, Rosmalawati S, Fukushima S, Kikuchi M, Hikichi Y, Kanda A, Takahashi A, Kiba A, Ohnishi K, Ichinose Y, Taguchi F, Yasuda C, Kodama M, Egusa M, Masuta C, Sawada H, Shibata D, Hori K, Watanabe Y. 2005. Catalog of Micro-Tom tomato responses to common fungal, bacterial, and viral pathogens. *Journal of General Plant Pathology* **71**: 8-22.

Tang WQ, Brady SR, Sun Y, Muday GK, Roux SJ. 2003. Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport. *Plant Physiology* **131**:147-154.

Weerasinghe RR, Swanson SJ, Okada SF, Garrett MB, Kim S-Y, Stacey G, Boucher RC, Gilroy S, Jones AM. 2009. Touch induces ATP release in *Arabidopsis* roots that is modulated by the heterotrimeric G-protein complex. *FEBS Letters* **583**:2521-2526.

Wu J, Steinebrunner I, Sun Y, Butterfield T, Torres J, Arnold D, Gonzalez A, Jacob F, Reichler S, Roux SJ. 2007. Apyrases (nucleoside triphosphate-diphosphohydrolases) play a key role in growth control in Arabidopsis. *Plant Physiology* **144**:961-975

Wu S, Wu J. 2008. Extracellular ATP-induced NO production and its dependence on membrane Ca^{2+} flux in *Salvia miltiorrhiza* hairy roots. *Journal of Experimental Botany* **59**:4007-4016.

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